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Conjugate for Mediating Cell-Specific, Compartment-Specific or Membrane-Specific Transport of Active Substances

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The present invention relates to conjugates for mediating cell-specific, compartment-specific or membrane-specific transport of active substances. The invention also relates to methods of producing said conjugates and their use.

As is known, cellular membrane systems are largely impermeable to many substances (e.g. nucleic acids, proteins, chemical substances) which shall be introduced into a cell from outside. For the introduction of nucleic acids it is possible to penetrate cell membranes by physical processes (transfection in the case of eukaryotes, transformation in the case of prokaryotes) and biological processes (infection). In the case of transformation, *i.e.* the direct take-up of the naked nucleic acid by the cell, the cells are treated beforehand. Various methods are available to produce these "competent cells". Most methods are based on the observations made by Mandel and Higa (J. Mol. Biol. 53, pages 159-163 (1970)) who were the first to show that it is possible to substantially increase the yields occurring when lambda-DNA is taken up by bacteria in the presence of calcium chloride. This method was used successfully for the first time by Cohen *et al.* (Proc._Natl. Acad. Sci. U.S.A. 69, pages 2210-2114 (1972)) for plasmid DNA and has been improved by many modifications. Another transformation method is based on the observation that high-frequency alternating-current fields can break up cell membranes (electroporation). This technique can be utilized to insert naked DNA not only in prokaryotic cells but also

in eukaryotic cell systems (Weaver *et al.*, J. Cell Biochem. 51, pages 426-435 (1993)). Two very mild methods of introducing DNA into eukaryotic cells were developed by Sikes *et al.* (Hum. Gen. Therap. 5, pages 837-840 (1994)) and Yang *et al.* (Proc. Natl. Acad. Sci U.S.A. 87, pages 9568-9572 (1990). They are based on the direct injection of the DNA into single cells (microinjection) and on the bombardment of a cell population using microprojectiles of tungsten on the surface of which the corresponding nucleic acid was bound (gene gun), respectively. In a progress parallel to the physical transformation of cells, biological infection methods have proved their efficiency. They comprise in particular the viral introduction of nucleic acids into cells (Chatterjee *et al.*, Science 258, pages 1485-1486 (1992); Cossett and Rusell, Gene Therapy 3, pages 946-956 (1996); Bilbao *et al.*, FASEB J. 11, pages 624-634 (1997)) and the liposome-mediated lipofection (Bennett *et al.*, J. Drug Targeting 5, pages 149-162 (1997)). Reference is also made to standard methods of the liposomal transport (Gao and Huang, Gene Therapy 2, pages 710-722 (1995); Akhtar *et al.*, Nucl. Acid. Res. 19, pages 5551-5559 (1991)) and poly-L-lysine formation (Leonetti *et al.*, Bioconj. Chem. 1(2), page 149 (1990) of active substances to be able to transport them into cells.

Despite the above-listed plurality of methods of passing through the cellular membrane systems, there is no universal method serving for introducing different active substances into cells. All of the above-mentioned physical and biochemical methods are artificial and non-physiological unless they make use of cell-immanent mechanisms. It is presently not yet certain that viruses used as transport vehicles are free of toxicity. They are often not effective and, in addition, they are detected by the immune system.

It was therefore the object of the present invention to provide a possibility of permitting the site-directed and specific introduction of active substances into cells and compartments. The following demands must be complied with in this connection:

- universal applicability
- cell-specific, compartment-specific and membrane-specific introduction behavior
- high degree of effectiveness
- low immunogenicity
- minimization of the infection risk
- sufficiently long residence time.

This object is achieved by the subject matters defined in the claims.

The inventors developed a conjugate comprising the following components:

- a transport mediator for the cell membrane ("P"),
- a cell-specific, compartment-specific or membrane-specific address protein or peptide ("AP"), and
- an active substance to be transported ("W").

The conjugate according to the invention is preferably composed as follows:

P - AP - W

More preferably it comprises a spacer ("SP"):

P - AP - SP - W

The transport mediator for the cell membrane (abbreviated as "P" above) is a peptide or protein which can penetrate the plasma membrane. The length of this peptide or protein is not subject to limitation as long as it has the above property. Examples of "P" are derived preferably from the penetratin family (Derossi *et al.*, 1998, Trends Cell Biol. 8, pages 84-87) or are transportan or parts thereof (Pooga *et al.*, The Faseb Journal (1998), Vol. 12, page 68 *et seq.*), those of the penetratin family being preferred. An example of "P" is a penetratin having the following sequence:

NH_2-RQIKIWFQNRRMKWKK-
(NH_2-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys)

Further examples of the transport protein "P" are as follows:

Viral transport protein
PTD protein transduction domain (TAT/HIV-1)
1-letter code H_2N-YGRKKRRQRRR-COOH
3-letter code H_2N-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg

Bacterial transport molecule
TP protein transport domain TP(Eco)
1-letter code H_2N-MTRQTFWHRIKH-COOH
3-letter code H_2N-Met-Thr-Arg-Gln-Thr-Phe-Trp-His-Arg-Ile-Lys-His

The select "P" sequence is produced biologically (purification of natural transport mediator proteins or cloning and expression of the sequence in a eukaryotic or prokaryotic expression system), preferably synthetically,

e.g. according to the established Merrifield method (Merrifield, J. Am. Chem. Soc. 85: 2149, 1963).

The selection of the address protein or peptide (abbreviated as "AP" above) depends on the membrane or membrane system which has to be penetrated and the target compartment of the cell (cytoplasm, nucleus, mitochondria, chloroplast, endoplasmic reticulum) or the cell organelle which shall be reached. The length of this address peptide or protein is not subject to limitation as long as it comprises the property of ensuring a cell-specific, compartment-specific or membrane-specific transport. For the introduction of active substances, in particular nucleic acids, "APs" are generally used which contain a cell-specific, compartment-specific or membrane-specific recognition signal, directing the attached active substance to its site of action. There are the "APs" to chose from which can transport active substances in the presence or absence of a membrane potential. The pure address sequence is usually sufficient for a transport into the cell compartment. However, it is also possible to chose "APs" which have a cell-specific or compartment-specific peptidase cleavage site. In the most favorable case, this cleavage site lies within the signal sequence but it can also be attached thereto by additional amino acids to ensure the cleavage of the address sequence after the target compartment is reached. The select "AP" sequence is produced biologically (purification of natural transport mediator proteins or cloning and expression of the sequence in a eukaryotic or prokaryotic expression system), preferably synthetically, e.g. according to the established Merrifield method (Merrifield, J. Am. Chem. Soc. 85: 2149, 1963). Examples of address proteins or peptides are as follows:

Import into the ER	H_3N^+-Met-Met-Ser-Phe-Val-Ser-Leu-Leu-Leu-Val-Gly-Ile-Leu-Phe-Trp-Ala-Thr-Glu-Ala-Glu-Gln-Leu-Thr-Lys-Cys-Glu-Val-Phe-Gln-
Reimport into the ER	H_2N-Lys-Asp-Glu-Leu-COO^-
Import into the mitochondria	H_3N^+-Met-Leu-Ser-Leu-Arg-Gln-Ser-Ile-Arg-Phe-Phe-Lys-Pro-Ala-Thr-Arg-Thr-Leu-Cys-Ser-Ser-Arg-Tyr-Leu-Leu
Import into the nucleus	-Pro-Pro-Lys-Lys-Lys-Arg-Lys-Val
	H_3N^+-Pro-Lys-Lys-Lys-Arg-Lys-Val- (= **n**uclear **l**ocalisation **s**equence from SV40-T antigen)
Import into peroxisomes	H_2N-Ser-Lys-Leu-COO^-
Binding to the cell membrane	H_3N^+-Gly-Ser-Ser-Lys-Ser-Lys-Pro-Lys

Furthermore, the conjugate may optionally contain a spacer (abbreviated as "SP" above) which is preferably located between the address protein/peptide and the active substance to be transported. However, it may also be located additionally or alternatively between the transport mediator and the address protein. The spacer serves for eliminating or positively influencing optionally existing steric interactions between the components. For example, the spacer may be selected from: polylysine, polyethylene glycol (PEG),

derivatives of poly-methacrylic acid or polyvinyl pyrrolidone (PVP).

A redox cleavage site, e.g. -cysteine-S-S-cysteine-O-N-H-, is preferably present between the transport mediator and the address protein/peptide. The binding forming between transport mediator and address protein is a redox coupling (mild cell-immanent bond by means of DMSO; Rietsch and Beckwith, 1998, Annu. Rev. Gent 32, pages 163-84):

Cysteine-**S**H **S**H-cysteine ----> cystine-**S-S**-cystine

The active substance or active agent (abbreviated as "W" above) is not subject to limitations. It can be chosen freely, depending on the effect which shall be produced in a cell. The active substance may be a diagnostic agent and/or a therapeutic agent. The conjugate may also comprise more than one active substance. The active substance may optionally be labeled, e.g. radioactively, with a dye, with biotin/avidin, *etc*. The active substance may be a nucleic acid, a protein or peptide, a chemical substance, *etc*. The next ones are mentioned by way of example: cDNA, genomic DNA, complete genes, regulatory elements, transcription factors, molecular probes, oligonucleotides, mRNA, mTRNA, antisense RNA, antisense oligonucleotides, plasmids, viral DNA, synthetic nucleotides, PNA (peptide nucleic acids), single amino acids and their derivatives, peptides, proteins, monoclonal and/or polyclonal antibodies, pharmaceutical active substances, chemotherapeutic agents, dyes, sensitizers, particles.

The conjugate elements "P" and "AP" are preferably synthesized synthetically according to the Merrifield method (Merrifield, J. Am. Chem. Soc. 85: 2149, 1963). The coupling

of the other constituents (e.g. spacer and/or active substance) thereto is made by covalent chemical binding. The redox cleavage site is inserted chemically between "P" and "AP" by the above-mentioned redox coupling. There is also a covalent bond, preferably an acid amide bond, between an optionally present spacer and the active substance or the address protein and the active substance. Possible alternatives are ether or ester bonds, depending on the functional group(s) present in the substance to be conjugated.

The conjugate is preferably synthesized in the following steps:

1) separate peptide snythesis of "P", "AP" and, if applicable, the spacer (e.g. according to the Merrifield method)
2) covalent bond between "AP" and active substance, if applicable, with a spacer in between,
3) redox coupling of the product from step 2) with "P" by means of redox coupling (e.g. in water/DMSO)
4) purification (e.g. by means of HPLC).

The conjugates according to the invention have the advantage that irrespective of the kind and size of an active substance they can introduce it into cells and transport it into the desired cell compartment. Thus, an improvement of diagnostics and therapy in human and veterinary medicines and an application in scientific research can be anticipated. In partiuclar, the gene therapy can expect a boom on account of the conjugates according to the invention since complete genes including their regulatory elements

become transportable. However, all of the other active substances can also be transported more specifically to the site of action by means of the conjugates according to the invention, which reduces the occurrence of undesired side effects. It was found that conjugates up to 25 MDa can be introduced into the cell interior. Moreover, apoptosis is often triggered, which might be a desired effect. The conjugates according to the invention distinguish themselves by a universal usability on account of their cell-specific, compartment-specific and membrane-specific introduction behavior.

The invention is described in more detail by means of the attached figures:

Figure 1 shows a conjugate according to the invention;

Figure 2 shows a general diagram of the Fmoc synthesis;

Figure 3 shows the results of the fluorescence correlation spectroscopy measurement using AT1 cells
- A) conjugate concentration: 50 nM
  incubation period: 5 hours
- B) conjugate concentration: 5 nM
  incubation period: 5 hours
- C) conjugate concentration: 50 nM
  incubation period: 24 hours
- D) conjugate concentration: 5 nM
  incubation period: 24 hours;

Figure 4 shows the concentration-dependent and time-dependent transport of $^{\text{rhodamine110}}$(L)-penetratin/RPMI medium;

DU145 cells: incubation with 20 µM and 100 pM final concentration;

Figure 5 shows examples of conjugates according to the invention;

Figure 6 shows the production of PNA constructs;

Figure 7 shows the inhibition of the proliferation of AT-1 cells by introducing an anti-sense construct.

The invention is described in more detail by means of the following examples.

Example 1: Conjugate comprising a penetratin constituent, an NLS, a polylysine spacer and rhodamine

Regarding the composition of the conjugate reference is made to figure 1.

Penetratin: NH_2-RQIKIWFQNRRMKWKK-

NLS (nuclear localisation sequence): NH_2-PKKKRKV

Spacer (= $(Lys)_2$): $NH-CH_2-(CH_2)_3-CHNH_2-CO-NH-CH_2-(CH_2)_3-CHNH_2-CO-NH$

Penetratin sequence, NLS and spacer were synthesized separately according to the standard Fmoc method ("peptides", H.-D. Jakubke, *Chemie und Biologie Spektrum*, Akad. Verl. 1996, ISBN 3-8274-0000-7). The general diagram of the Fmoc synthesis is shown in figure 2. For synthesizing the different component sequences, the first Fmoc amino acid

(purchasable from Calbiochem GmbH, D-65796 Bad Soden, Germany) is initially attached to an insoluble polystyrene carrier resin via an acid-labile linker (= para-benzyl-oxybenzyl-alcohol-handle). Cleavage of the protecting group is achieved by treating the resin with 20 % piperidine in dimethylformamide. The second Fmoc amino acid is linked using a preactivated species (e.g. succinimide, pentafluorophenylester or p-nitrophenylester groups present in the amino acid constituents) or using *in situ* activation, this was done in each case after the protecting group was removed from the preceding amino acid by basic treatment. Each further amino acid is coupled analogously. Having synthesized the desired peptide, it is removed from the carrier by treating it with 95 % trifluoroacetic acid (TFA) + 5 % scavenger (e.g. triethylsilane), and the protecting groups are splitt off. The resulting crude peptides are purified by preparative HPLC on a YMC ODS-A 7A S-5µm reversed-phase column (20 x 250 mm) using an elution agent containing 0.1 % trifluoroacetic acid in water (A) or 60 % aqueous actonitrile (B). The peptides were eluted with a successive linear gradient from 25 % B to 60 % B within 40 minutes at a flow rate of 10 ml/min. The fractions corresponding to the purified peptides were lyophilized.

The purified peptide components are treated together with 20 % aqueous DMSO solution at room temperature for 5 hours, an oxidative coupling of the components resulting. For example, rhodamine 110 is coupled to the spacer as active substance to be transported. This is done by acid amide coupling at the free α-amino group of the lysine spacer. The complete conjugate is then purified by means of reversed-phase HPLC.

The further conjugates according to the invention were produced analogously:

$^{Alexa™}$(L)-PTD$^{(TAT/HIV-1)}$-S-S-(L)-NLS-KK$^{(rhodamine110)}$-PNA

$^{Alexa™}$(L)-TP$^{(1AOP/ECo)}$-S-S-(L)-NLS-KK$^{(rhodamine110)}$-PNA

PNA = NH_2-TTA AGG AGG CTC COOH (Example of active substance)
Alexa 350 = dye (Molecular Probes, U.S.)

Example 2: Introduction of a conjugate according to the invention into cells

AT-1 (rat prostate carcinoma) and DU-145 (human prostate carcinoma, ATCC HTB-81) cells were cultured in RPMI 1640, supplementd with 10 % FCS, 2 mM glutamine, 100 U/min. penicillin, 100 µg/ml streptomycin.

For fluorescence correlation spectroscopy (FCS) AT-1 or DU-145 cells are grown on slides for 24 hours. Having changed the medium using dyestuff-free RPMI 1640 (without phenol red), the penetratin-containing conjugate of Exmaple 1 (100 nM) is placed onto the cells using RPMI and incubated at 37°C and with 5 % CO_2 for 5, 24 or 48 hours. Thereafter, the conjugate-containing medium is removed and washed twice with 200 µl of dyestuff-free RPMI and then measured by means of FCS. Laser excitation takes place at 488 nm and emission at 538 nm.

The conjugate is pursued on its way into the nucleus. For this, a cell is selected and focused under the light microscope. Having focused and set the laser, 100-µm steps are used for passing through the cells, and fluorescence is measured in the form of flashes by photomultipliers. Here, large molecules and small molecules migrate at differing speeds. The number of molecules diffusing in an area of 100 µm each is detected. In this way, the size of the diffused

molecules can be determined by means of the duration of the signal. The accompanying diagram is shown in figure 3.

In another experiment, the kinetics by which the conjugate reaches the cytoplasm is determined by the same method. The AT-1 cells were again attached for 24 hours. The medium containing the conjugate was used as described above. However, in this case, the fluorescence signal was immediately measured by FCS.

FCS clearly showed a strong accumultation on the cell membrane after an incubation period of 5 hours. Diffusion could not be detected. Only minor amounts of conjugate could be found in the cell menbrame after an incubation period of 24 hours. Attention was then attracted by an accumulation in the nucleus which became even more intense within the observation period of 48 hours.

For the purpose of control conjugates were used in which rhodamine 110 was only bound to either penetratin or NLS. They did not show the above-described effect of nucleus accumulation. If they succeeded at all in penetrating the cell, the conjugates were stopped at the cell membrane of nuclear envelope where they accumulated.

As described analogously above, all of the conjugates produced in Example 1 were studied as regards their time-dependent intracellular transport into the cytoplasm (Z) or the nucleus (N). However, differing from the above-mentioned incubation periods the incubation periods were 1, 3, 6, 10 and 24 hours. The results are shown in Table 1.

Example 3: Concentration-dependent transport

The purpose of the study was to determine to what extent the concentration of the transport peptide $^{\text{rhodamine110}}$(L)-penetratin/RPMI medium influences the cellular and nucleus-directed transport in terms of time as well. A comparison was made between the fluorescence of 20 μM and 100 pM final concentration of $^{\text{rhodamine110}}$(L)-penetratin/RPMI medium. For this purpose, DU-145 cells were incubated at the indicated concentrations for 1, 6, 12, 24 and 48 hours. Thereafter, washing was carried out three times with RPMI (without penetratin), once with PBS and again with RPMI. Having provided the cells with slide covers, fluorescence was determined directly afterwards by means of CLSM (confocal laser scanning microscopy). The results are shown in figure 4. It follows therefrom that at a high concentration of over 20 μM a non-specific transport takes place, which suggests cytotoxicity. However, in a lower concentration there is specific transport into the cytoplasm.

Example 4: Inhibition of the proliferation of AT-1 cells by introducing an anti-sense construct

Peptide-conjugate constructs according to figure 6 were produced using the method described in Example 1 analogously. Here, the active substance was in one case a PNA having the sequence NH_2-TAC TGC GAC TCC GG-COOH (anti-sense with respect to rats P2 promoter c-myc = PNA_{AS}) and then a non-sense (random) sequence having the nucleotide sequence NH_2-TTA AGG AGG CTC-COOH (=PNA_{NS}).

AT-1 cells were cultured in RPMI 1640, supplemented using 10 % FCS, 2 mM glutamine, 100 U/min. penicillin,, 100 μg/ml streptomycin.

AT-1 cells are grown on slides for 24 hours. Having changed the medium using dyestuff-free RPMI 1640 (without phenol red), the conjugates (100 nM) are placed onto the cells with RPMI each and incubated at 37°C and with 5 % CO_2 for 24, 48, 72 or 96 hours. Thereafter, the conjugate-containing medium is removed and washed twice with 200 µl dyestuff-free RPMI. The cell number of AT-1 cells is determined by means of the Coulter counting method.

Untreated AT-1 cells were used as a control. Unligated PNA_{AS} represents another control. As described analougously above, these controls were incubated with the AT-1 cells.

The result of this experiment is shown in figure 7. The proliferation of AT-1 was only inhibited after the administration of the anti-sense construct, *i.e.* this shows clearly that penetration of the nucleus where the anti-sense sequence can display the desired effect takes only place by means of the construct according to the invention. Unligated anti-sense sequence is as ineffective as the control or a construct which cannot hybridize with one of the AT-1 sequences.